Human Immunodeficiency Virus (HIV) reverse-transcriptase region (pol gene) amplification & sequencing.

Created: Oct 26, 2010; **Last modified**: Mar 24, 2021, **Version:** 2.0

This protocol describes the nested RT-PCR approach to reverse transcriptase region (pol gene) amplification and sequencing using integrated proviral DNA or viral RNA as template. This protocol was created to allow for the characterisation of anti-retroviral drug resistance-mutations. The nested strategy allows for better success at sequencing from samples having either low quality, concentration of template or viral load (in the case of RT-PCR). The fragment thus generated partially overlaps on the 5' end with the 3' end of the protease (Prot) encoding fragment, allowing together with that of the integrase encoding fragment for the assembly of full-pol region contigs.

Oligonucleotides

Name		PCR	Sequence*	bp	%GC	Tm	Position †	Amplicon	Ref.
RT-a	RT-FO2	1st RTa	5'-ggA-AAC-CAA-AAA-TgA-TAg-gRg-gAA-3'	24	39.6	54.4	2377-2400	1416	1
	RT-RO3		5´-TCC-CAY-TCA-ggA-ATC-CAg-gT-3´	20	52.5	56.7	3774-3793		
	RT-FI2	2nd RTa	5'-Agg-TAC-AgT-ATT-AgT-Agg-ACC-TAC-3'	24	41.7	52.4	2468-2491	1058	
	RT-RI3		5'- AYY-AAg-TCT-TTT-gAT-ggg-TC-3'	20	40	50.0	3507-3526		
RT-b	RT-FO3	1st RTb	5'-CTg-gAC-TgT-CAA-TgA-YAT-ACA-RA-3'	23	39.1	52.3	3302-3324	1102	
	RT-RO2		5'-CCT-ggA-CTA-CAg-TCT-ACT-TgT-CC-3'	23	52.2	56.2	4382-4404		
	RT-FI3	2nd RTb	5'- gTC-AAT-gAY-ATA-CAR-AAR-TTA-gTR-gg-3'	26	34.6	51.8	3309-3334	986	
	RT-RI2		5'-CAT-TgC-TCT-CCA-ATT-RCT-gTg-3'	21	45.2	53.1	4275-4295		

* Reverse oligonucleotide primer sequences given in this table are the reverse-complement of the sequence present in the alignments and as they should be ordered for synthesis.

† Primer binding sites given on table are based on HXB2 reference sequence.

RT-PCR components and conditions

Using Vivantis M-MuLV RT enzyme (Cat: ME2305) and Vivantis Taq DNA Pol (Cat: PL1202).

First strand synthesis (RT)

	cf	1x
dH_2O	---	2.5 µL
10 µM Forward oligo	1.125 µM	2.3 µL
10 µM Reverse oligo	1.125 µM	2.3 µL
10 mM dNTPs 10 mM	250 µM	0.5 µL
RNA	---	10 µL
		vf: 17.6 µl

➔

RT-1 program in Axygen TC-1		
Total time: 6 min		
95 ºC	2 min	1 cycle
4 ºC	2 min	

↓

RT Buffer	1x cf	2 µL
RT Enzyme 250 IU/µl	5 IU/µL	0.4 µL
		vf: 20 µl

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Run RT-2 program in Axygen TC-1 ➔

RT-2 program in Axygen TC-1		
Total time: 1:12 hrs		
38 ºC	60 min	1 cycle
95 ºC	5 min	
4 ºC	5 min	

↓

1st Polymerase Chain Reaction (PCR) both fragments

	cf	1x
dH_2O	C*f*	6.02
10x Buffer PCR	1x	1.25
$MgCl_2$ 50 mM	1.50 mM	0.38
dNTPs 10 mM	200 µM	0.25
Oligos 10 µM	400 nM	0.50
Taq (Vivantis) 5 UI/µL	0.04 UI/µL	0.10
DNA	-	4.00
		vf: 12.5 µl

➔

Run 1st PCR in Axygen cycler		
Total time: 1:30 hrs		
95 ºC	2 min	
95 ºC	30 sec	30 cycles
55[a] /54[b] ºC	45 sec	
72 ºC	1 min	
72 ºC	5 min	

↓

2nd Polymerase Chain Reaction (PCR) both fragments

	cf	1x
dH_2O	C*f*	9.02
10x Buffer PCR	1x	1.25
$MgCl_2$ 50 mM	1.50 mM	0.38
dNTPs 10 mM	200 µM	0.25
Oligos 10 µM	400 nM	0.50
Taq (Vivantis) 5 UI/µL	0.04 UI/µL	0.10
1st PCR product	-	1.00
		vf: 12.5 µl

➔

Run 2nd PCR in Axygen cycler		
Total time: 1:30 hrs		
95 ºC	1 min	
95 ºC	30 sec	30 cycles
51[a] / 54[b] ºC	30 sec	
72 ºC	1 min	
72 ºC	5 min	

Notes

1. Clean workbench with 0.1% NaOCl 0.1% followed by 70% Ethanol before and after work.
2. Preparation of RT mastermix should only be performed in the RT-PCR room.
3. Preparation of PCR mastermix and addition of sample DNA should only be performed in the pre-PCR enclosure or area.
4. Addition of positive template DNA should be performed on instrument (post-PCR) area.
5. All mastermixes should be prepared on ice to prevent excess evaporation.
6. Vortex and spin all mastermixes before and after aliquoting to PCR tubes.

References

1. Swanson P, Devare SG, Hackett J Jr. Molecular characterization of 39 HIV isolates representing group M (subtypes A-G) and group O: sequence analysis of gag p24, pol integrase, and env gp41. AIDS Res Hum Retroviruses. 2003 Jul;19(7):625-9. PubMed PMID: 12921095.

Revision history

1.0 Original document.

2.0 Changes to document format only.